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THE ADAPTATION OF THE HEIST-LACY METHOD FOR DETERMINING THE BACTERICIDAL ACTIVITY OF WHOLE BLOOD FOR CHEMOTHERAPEUTIC INVESTIGATIONS

STUDIES IN THE CHEMOTHERAPY OF BACTERIAL INFECTIONS. I

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In chemotherapeutic investigations in bacterial infections, test tube bactericidal tests are of service for detecting evidences of specific bacteriotropism and for furnishing "leads" in the preparation of new compounds; when these tests are conducted with serum or blood they may indicate the important influence of these substances on bactericidal activity, but they are no index to the degree of bactericidal activity occurring in vivo when administered intravenously to infected living animals and do not measure the degree of organotropism or toxicity for body cells.

Tests conducted in vivo with experimental infections may yield negative results because the drug may be of such toxicity or high organotropism that an amount sufficient to exert a demonstrable degree of bactericidal activity or parasitropism cannot be administered; notable examples are various mercurial compounds possessing marked bactericidal activities in the test tube even in serum and blood, but proving too toxic for the living animal to permit the administration of an amount sufficient to influence materially a generalized infection or bacteremia. Even if a compound may be administered in an amount sufficient to increase the bactericidal activity of the blood for a certain bacterium, the micro-organisms may proliferate so rapidly or gain access to the blood in such numbers that the feeble but positive bactericidal activity of the compound may be masked and undetermined. This is apparently true of ethylhydrocuprein (optochin) hydrochlorid in the treatment of lobar pneumonia, as shown by the studies of Moore and Chesney.[1]

Experimentally produced local infections of serous cavities provide more sensitive lesions for chemotherapeutic studies in vivo than bacteremias or generalized infections. Kolmer and Idsumi[2] found that

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[1] Arch. Int. Med., 1917, 19, p. 611; 1918, 21, p. 659.

[2] Jour. Infect. Dis., 1920, 26, p. 355.

subtoxic amounts of ethylhydrocuprein (optochin) hydrochlorid injected into the subarachnoid space of the spinal cord may favorably influence the course of pneumococcus meningitis in rabbits; in a similar study with Sands [3] in experimental pneumococcus pleuritis of guinea-pigs, the results were more decisive. These investigations have indicated that subtoxic amounts of ethylhydrocuprein hydrochlorid injected into these cavities may be sufficient to exert marked pneumococcidal activity even when the drug is diluted with the inflammatory exudates. The degree of dilution, however, is probably never as great as when the drug is introduced into the blood stream and hence exerts more marked curative activity in an infected cavity before being absorbed than is possible in the treatment of a bacteremia or generalized infection.

In vivo experiments are usually prolonged, laborious and expensive and require frequent repetition before reliable results are obtained. Of greater importance, however, is the fact that minor degrees of specific parasitropism of new compounds may not be elicited for the reasons previously mentioned, and thereby fail to furnish data for the guidance of chemotherapeutic studies. For this reason a combined in vivo-in vitro test may prove of value when conducted by administering the drug in sublethal or subtoxic amounts to animals, securing small amounts of blood at intervals and measuring the bactericidal activity of the whole blood or serum in vitro. By means of plating methods Moore and Chesney [1] were able to show that ethylhydrocuprein (optochin) hydrochlorid administered intravenously, intramuscularly or orally may raise the pneumococcidal activity of the serum even though the drug is too toxic to permit the administration of an amount sufficient to raise this bactericidal activity to the range of therapeutic activity in pneumococcus pneumonia.

In a search for a reliable technic for measuring the bactericidal activity of the whole blood adaptable for use on a large scale in the course of chemotherapeutic investigations with the lower animals, we have given the Heist-Lacy method an extensive trial, and the purpose of this paper is to summarize the results observed.

The technic has been described by Heist, S. Solis-Cohen and M. Solis-Cohen.[4] The essential feature of the method, suggested by Benjamin F. Lacy, is that a capillary glass tube is filled, by capillary attraction, up to a fixed mark, with a broth culture of the bacterium, and then emptied. A certain number of bacteria remain sticking to the wall of the tube. Blood as it comes from capillary or vein is allowed to flow up the tube to the mark, and the tube is then sealed and incubated. If the blood has no bactericidal action the bacteria which have remained on the wall of the tube find themselves in a favorable medium and multiply rapidly: If it is bactericidal, they are killed and no growth results. Readings are made by blowing out the contents of the tube on a glass slide and staining and examining them under the microscope. By combining several capillary tubes into one many-stemmed pipet, modeled after the one Wright uses for estimating the coagulation time of the blood, and by using a series of ascending dilutions of broth culture, an approximate quantitative value may be given to the test.

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[3] Jour. Exper. Med., 1921, 33, p. 693.

[4] Jour. Immunology, 1918, 3, p. 261.

By means of this method, which is remarkably simple as compared with the usual plating bactericidal tests and employs whole blood instead of serum, Heist, Cohen and Cohen[4] were able to demonstrate the high pneumococcidal activity of the blood of the pigeon in contradistinction to the feeble or absent pneumococcidal activity of the blood of the rabbit. They were also able to measure the degree of active immunity resulting from immunization of rabbits with pneumococci of different types.[5] Matsunanni and Kolmer found that the test elicited varying degrees of natural immunity to virulent meningococci,[6] corroborating the observations of Heist, Cohen and Cohen, that the results of this in vitro test tend to run parallel with the degree of immunity elicited by injecting animals with virulent cultures of bacteria.

EXPERIMENTAL

Our experiments were conducted by determining the bactericidal activity of the blood of normal rabbits before and at varying intervals after the administration of several compounds.

Two micro-organisms were employed: a highly virulent type 1 pneumococcus and a culture of Staphylococcus aureus. Heist has emphasized the importance of using young broth cultures of virulent pneumococci; our experiments were conducted with 18-hour cultures in the hormone broth described by Huntoon. As shown in the tables, 5 dilutions made with hormone broth were employed: 1:10, 1:100, 1:1,000, 1:10,000 and 1:100,000.

As stated, the test is conducted with the micro-organisms adhering to the interiors of the capillary tubes; the actual numbers vary according to the density of the parent growth but with a uniform broth remains fairly constant. In order to minimize this error and render different experiments more comparative, Heist has adopted the practice of making large steps in preparing the dilutions of culture.

[5] Heist, G. D., and Cohen, S. Solis: Ibid., 1919, 4, p. 147.

[6] Ibid., 1918, 3, p. 201.

By washing out the tubules with broth and plating these fluids Heist has found that the actual numbers of viable staphylococci adhering to the interiors of the capillary tube were approximately as follows:

	Staphylococcus
Undiluted	1900
1: 10	500
1: 100	100
1: 1,000	20
1: 10,000	8

The majority of the experiments were conducted with ethylhydrocuprein (optochin) hydrochlorid, other quinine compounds such as the bisulphate, hydrochlorid and quinine and urea hydrochlorid; mercurophen (sodium oxymercury-ortho-nitro phenolate) and mercuric chlorid as a control on the results observed with mercurophen.

Healthy adult rabbits weighing from 1,800 to 2,400 gm. were employed. Blood was secured from ear veins after cleansing and contaminations did not occur confirming the observations of Heist, Cohen and Cohen[4] that nonpathogenic bacteria do not grow in whole blood unless comparatively large numbers are seeded.

Preliminary tests were always made, but the whole blood of rabbits was found practically without bactericidal activity for the cultures of pneumococci and staphylococci employed in this study.

The medicaments were administered intravenously, subcutaneously and by stomach tube in doses according to body weight. The bactericidal tests were then repeated at varying intervals. In some experiments the medicaments were given once daily for 4 days, the bactericidal tests being conducted 24 hours after each administration and just before the succeeding dose. After the fourth dose daily bactericidal blood tests were conducted until the influence of the medicament had disappeared. In every experiment culture controls with broth were set up each day and always yielded heavy growths.

RESULTS OBSERVED WITH OPTOCHIN (ETHYLHYDROCUPREIN) HYDROCHLORID

(*a*) *Single Intravenous Injection.*—As shown in tables 1 and 2, the intravenous injection of 10 and 20 mg. of ethylhydrocuprein hydrochlorid per kilo of body weight imparted a decided pneumococcidal activity to the blood of rabbits. These amounts were approximately ⅙ to ¼ the highest tolerated doses by slow intravenous injection.

This increased bactericidal activity was apparent within a few minutes after each injection, but appeared to increase slightly during the following 24 hours, to be followed by a sharp decline.

Duplicate tests made at the same time with a culture of Staphylococcus aureus showed a slight increase in bactericidal activity particularly with the 20 mg. dose of the drug. These effects were apparent, however, only with the higher dilutions of the culture, the greater bactericidal activity of the drug for the pneumococcus standing out in sharp contrast.

(*b*) *Multiple Intravenous, Subcutaneous and Oral Administrations.*—The pneumococcidal activity of the blood of a series of rabbits was tested on each of 2 succeeding days and found practically absent even with 1: 100,000 dilutions of culture. Ethylhydrocuprein hydrochlorid was then administered intravenously in a dose of 10 mg. per kilo; subcutaneously in a dose of 20 and 30 mg. per kilo and by stomach tube in a dose of 25 and 40 mg. per kilo. The drug was given once a day for 4 days and the blood tests repeated daily. The results are summarized in table 2.

Twenty-four hours after the first dose the whole blood had acquired well defined pneumococcidal properties even after the oral administration of the drug, which confirms the observations of Moore and Chesney.[1] With the succeeding doses the bactericidal effects became more marked. After the last dose (fourth) these bactericidal activities promptly diminished and were usually absent two or three days later.

TABLE 1

THE INFLUENCE OF ONE INTRAVENOUS INJECTION OF ETHYLHYDROCUPREIN HYDROCHLORID IN A DOSE OF 0.01 GM. PER KILO ON THE BACTERICIDAL ACTIVITY OF THE BLOOD OF RABBITS FOR PNEUMOCOCCI AND STAPHYLOCOCCI

Blood Tests	Pneumococcus Type 1					Staphylococcus aureus				
	1:10*	1:100	1:1000	1:10,000	1:100,000	Undil.*	1:10	1:100	1:1000	1:10,000
Before injection	++	++	++	++	++	++	++	++	++	++
Immediately after	++	++	—	—	—	++	++	++	++	++
1 hour after	++	—	—	—	—	++	++	++	++	++
4 hours after	++	+	—	—	—	++	++	++	++	++
24 hours after	++	—	—	—	—	++	++	++	—	—
48 hours after	++	++	++	++	++	++	++	++	++	++
Culture controls	++	++	++	++	++	++	++	++	++	++

* Dilutions of 18-hour broth cultures.
+ = heavy growth; + = slight growth; — = no growth.

TABLE 2

THE INFLUENCE OF MULTIPLE ADMINISTRATIONS OF ETHYLHYDROCUPREIN HYDROCHLORID ON THE PNEUMOCOCCIDAL ACTIVITY OF THE BLOOD OF RABBITS

Blood Tests	Intravenously; 10 Mg. per Kilo					Subcutaneously; 20 Mg. per Kilo					By Mouth; 40 Mg. per Kilo				
	1: 10*	1: 100	1: 1,000	1: 10,000	1: 100.000	1: 10	1: 100	1: 1.000	1: 10,000	1: 100,000	1: 10	1: 100	1: 1.000	1: 10,000	1: 100,000
First preliminary	++	++	++	++	+	++	++	++	++	++	++	++	++	++	++
Second preliminary	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
24 hours after first dose	+	—	±	—	—	+	—	—	—	—	+	+	—	—	—
24 hours after second dose	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—
24 hours after third dose	—	—	—	—	—	—	—	—	—	—	++	++	—	—	—
24 hours after fourth dose	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
48 hours after fourth dose	++	++	++	++	++	++	++	+	+	+	++	++	+	—	—
72 hours after fourth dose	++	++	++	+	—	++	++	++	+	±	++	+	+	—	—
96 hours after fourth dose	++	++	++	++	++	++	++	++	++	+	++	++	++	++	++
Culture controls	++	++	++	++	++	++	++	++	++	+	++	++	++	++	++

* Dilutions of 18-hour broth cultures.

RESULTS OBSERVED WITH OTHER CINCHONA COMPOUNDS

As shown by Cohen, Kolmer and Heist[7] and others, many of the ordinary compounds of quinine are pneumococcidal, particularly in vitro. None, however, have been found equal to ethylhydrocuprein hydrochlorid either in vitro or in protective and curative tests with infected mice.

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[7] Jour. Infect. Dis., 1917, 20, p. 272.

In order to determine the value of the Heist-Lacy method for eliciting varying degrees of bactericidal activity, three quinine compounds have been tested and the results compared with those observed with ethylhydrocuprein hydrochlorid.

The compounds were administered intravenously in a dose of 10 mg. per kilo; subcutaneously in a dose of 30 mg. per kilo and by stomach tube in a dose of 25 mg. per kilo. (The results are illustrated in table 3.)

All of the 3 compounds employed, namely, quinine and urea hydrochlorid, quinine bisulphate and quinine hydrochlorid, imparted a definite pneumococcidal activity to the blood by all 3 routes of administration. These effects were slightly better after intravenous injection than after subcutaneous and oral administration. The effects produced by the 3 drugs were closely similar although the hydrochloride, which contains about 81% of the alkaloid, was slightly superior to the bisulphate and quinine and urea hydrochlorid which contain about 58% alkaloid. All 3 were inferior to ethylhydrocuprein hydrochlorid which contains about 90% of alkaloid.

TABLE 3

THE INFLUENCE OF MULTIPLE ADMINISTRATIONS OF QUININE HYDROCHLORIDE ON THE PNEUMOCOCCIDAL ACTIVITY OF THE BLOOD OF RABBITS

Blood Tests	Intravenously; 10 Mg. per Kilo					Subcutaneously; 30 Mg. per Kilo					By Mouth; 25 Mg. per Kilo				
	1: 10*	1: 100	1: 1,000	1: 10,000	1: 100,000	1: 10	1: 100	1: 1,000	1: 10,000	1: 100,000	1: 10	1: 100	1: 1,000	1: 10,000	1: 100,000
First preliminary	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+
Second preliminary	++	++	++	+	+	++	++	++	++	++	++	++	++	++	++
24 hours after first dose	++	++	++	+	+	++	++	++	++	0	++	++	++	++	++
24 hours after second dose	+	—	—	—	—	+	+	+	—	—	—	—	—	—	—
24 hours after third dose	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—
24 hours after fourth dose	++	++	++	++	+	++	++	+	+	—	++	+	+	—	—
48 hours after fourth dose	++	++	++	++	++	++	++	++	++	++	++	++	++	+	—
72 hours after fourth dose	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Culture controls	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++

* Dilutions of 18-hour broth cultures of pneumococcus type 1.

It was interesting to find, however, that the Heist-Lacy method is capable of yielding approximately quantitative results and that the administration of the common quinine compounds appreciably increases the pneumococcidal activity of the blood, even when administered by mouth.

RESULTS WITH MERCUROPHEN AND MERCURIC CHLORID

Mercurophen, which is a compound of mercury introduced by Schamberg, Kolmer and Raiziss,[8] possesses an extremely high and selective bactericidal activity in vitro for cocci, especially for staphylococci. For members of the typhoid-colon group of bacilli its bactericidal activity is about the same or slightly inferior to mercuric chlorid.

As reported by Schamberg, Kolmer and Raiziss,[8] the intravenous injection of mercurophen into rabbits in sublethal amounts (0.0015 gm. per kilo) was found to increase appreciably the bactericidal activity of the blood for a few hours for staphylococci, pneumococci and B. typhosus, whereas the administration of mercuric chlorid and other mercurial compounds usually failed to increase to the same degree the bactericidal activity of the whole blood of rabbits for these micro-organisms.

TABLE 4

THE INFLUENCE OF ONE INTRAVENOUS INJECTION OF MERCUROPHEN IN A DOSE OF 0.0008 GM. PER KILO ON THE BACTERICIDAL ACTIVITY OF THE BLOOD OF RABBITS FOR PNEUMOCOCCI AND STAPHYLOCOCCI

Blood Tests	Pneumococcus Type 1					Staphylococcus aureus				
	1:10*	1:100	1:1000	1:10,000	1:100,000	1:10*	1:100	1:1000	1:10,000	1:100,000
Before injection.........	++	++	++	++	++	++	++	++	+	+
Immediately after.......	++	++	++	+	+	++	+	—	—	—
1 hour after.............	++	++	++	++	+	++	++	+	—	—
4 hours after	++	++	++	++	+	+	±	—	—	—
24 hours after	++	++	++	++	+	++	++	+	+	+
48 hours after............	++	++	++	++	+	++	++	++	++	++
Culture controls.........	++	++	++	++	++	++	++	++	++	+

* Dilutions of 18-hour broth cultures.

TABLE 5

THE INFLUENCE OF ONE INTRAVENOUS INJECTION OF MERCUROPHIN IN A DOSE OF 0.0008 FOR PNEUMOCOCCI AND STAPHYLOCOCCI

Blood Tests	Intravenously; 5 Mg. per Kilo*					Subcutaneously; 10 Mg. per Kilo					Orally; 20 Mg. per Kilo				
	1: 10†	1: 100	1: 1,000	1: 10,000	1: 100,000	1: 10	1: 100	1: 1.000	1: 10,000	1: 100 000	1: 10	1: 100	1: 1,000	1: 10,000	1: 100,000
First preliminary.	++	++	++	++	+	++	++	++	++	++	++	++	++	++	++
Second preliminary............	++	++	++	++	+	++	++	++	++	++	++	++	++	++	++
24 hours after first dose........	++	++	+	—	—	++	++	++	—	—	+	—	—	—	—
24 hours after second dose.....	++	+	+	—	—	+	+	—	—	—	+	—	—	—	—
24 hours after third dose.......	+	—	—	—	—	+	+	+	—	—	+	—	—	—	—
24 hours after fourth dose.....	++	+	—	—	—	++	++	+	—	—	++	+	—	—	—
48 hours after fourth dose.....	++	++	++	++	++	++	++	++	++	++	++	+	+	+	+
72 hours after															
Culture controls..	++	++	++	++	++	++	++	++	+	—	++	++	++	++	++
fourth dose.....	++	++	++	++	++	++	+	++	++	++	++	++	++	++	++

* Rabbit died 96 hours after fourth dose.

† Dilutions of 18-hour broth cultures of pneumococcus type 1.

Similar results were observed in this study. The intravenous injection of 0.0008 gm. mercurophen per kilo, which is about ⅛ the highest tolerated dose, increased the bactericidal activity of the blood for staphylococci but not for pneumococci (table 4). A similar dose of mercuric chlorid, which corresponded to about ⅕ the highest tolerated dose, slightly raised the bactericidal activitiy of the blood for pneumococci.

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[8] Ibid., 1919, 24, p. 547.

As stated, in vitro tests have shown that mercurophen has a specially high bacteriostatic and bactericidal effect on staphylococci, and this superiority over mercuric chlorid was apparent in the results of the Heist-Lacy tests.

Mercurophen, however, also possesses pneumococcidal activity when administered to rabbits intravenously, subcutaneously and by mouth. But large amounts are required to produce these effects.

Table 5 shows the results observed when multiple doses were given intravenously, subcutaneously and by mouth.

The amounts of mercurophen administered were approximately one-half the highest tolerated dose (not the MLD). As shown in this table, appreciable effects were apparent after the first dose, which increased after subsequent doses but rapidly disappeared when the administration ceased. The amounts of mercuric chlorid administered were practically the highest tolerated doses; a slight increase of the pneumococcidal activity of the blood was apparent with the higher dilutions of culture, but the results were more irregular and less marked than observed with mercurophen.

SUMMARY AND CONCLUSIONS

In chemotherapeutic investigations in bacterial infections four procedures may be employed for evaluating a compound: (1) bacteriostatic and bactericidal tests in vitro; (2) a combined in vivo-in vitro test consisting of the administration of the compound in subtoxic amounts to animals and testing the bactericidal activity of the whole blood or serum at intervals; (3) the treatment of experimentally produced localized infections, such as meningitis and pleuritis, and (4) the treatment of experimentally produced generalized infections.

The Heist-Lacy method is a simple method for determining the bactericidal activity of whole blood at intervals after the administration of a drug and yields approximately quantitative results. If rabbits are employed, the test micro-organism should be one capable of surviving and proliferating in the blood of these animals, in order to elicit more clearly the possible bactericidal activity imparted by the drug under study.

The administration of ethylhydrocuprein (optochin) hydrochlorid to rabbits by intravenous, subcutaneous and oral routes results in rendering the whole blood decidedly pneumococcidal. Similar but much less marked results followed the administration of other quinine compounds as the bisulphate, hydrochlorid and quinine and urea hydrochlorid. The bactericidal properties of the blood rapidly disappeared after the administration of these compounds had ceased.

By means of the Heist-Lacy method it was found that the administration of large amounts of mercurophen appreciably raised the bactericidal activity of the blood of rabbits for staphylococci and pneumococci, especially the former. The administration of mercuric chlorid was followed by slight and irregular increase of the bactericidal activity of the blood of living rabbits for these micro-organisms.

The Heist-Lacy method has been found less susceptible to contamination and less laborious than plating methods for measuring the bactericidal activity of whole blood. Since only a few drops of blood are required it permits the use of small animals and of repeated tests.